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Baculovirus-mediated Expression of a *Manduca sexta* Chitinase Gene: Properties of the Recombinant Protein*

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We constructed a recombinant nonoccluded baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), containing a 1.8 kb DNA fragment from a *Manduca sexta* (tobacco hornworm) chitinase cDNA under the control of the polyhedrin gene promoter. When *Spodoptera frugiperda* (fall armyworm) cells (SF9) were infected with this recombinant virus, a protein with an apparent molecular weight of 85 kDa was secreted into the culture medium. This protein hydrolyzed chitin and cross-reacted with a polyclonal antibody to *M. sexta* molting fluid chitinase. Tunicamycin treatment of infected SF9 cells and subsequent western blot analysis indicated that the secreted enzyme was a glycoprotein. GC-MS analysis revealed that carbohydrate accounted for approximately 25% of the mass of glycoprotein. The recombinant chitinase and the molting fluid enzyme were indistinguishable by N-terminal sequencing, polyacrylamide gel electrophoresis and carbohydrate analysis, indicating that the recombinant protein was similar, if not identical, to the molting fluid enzyme. Analysis of the expression level of recombinant chitinase in SF9, SF21 and *Trichoplusia ni* (Hi-5) cell lines showed that the yields were in the order Hi-5 > SF21 > SF9. Chitinase accumulated in hemolymph after injection of fourth instar *M. sexta* and *S. frugiperda* larvae with recombinant virus. The median time for mortality of *S. frugiperda* fourth instar larvae infected with the recombinant virus was approximately 20 h shorter than that for insects infected with a wild type virus. The results support the hypothesis that insect chitinase has potential to enhance the insecticidal activity of entomopathogens.

Chitinase Baculovirus *Manduca sexta* *Spodoptera frugiperda* Insect cell lines Toxicity Glycoprotein Hemolymph

INTRODUCTION

Baculoviruses have been isolated from a number of insect pests and shown to be moderately effective as pest control agents (Vail, 1993). With increasing pressure from regulatory agencies to reduce environmental impacts of pest control practices and with the development of widespread resistance to chemical pesticides, baculoviruses are becoming more attractive as potential alternatives to conventional insecticides. However, slow rates of killing of infected larvae and narrow host ranges are major drawbacks to their development as commercial products. Enhancing the pesticidal activity of these viruses would aid in their utilization as biocontrol agents. This may be possible by introducing one or more genes with insecticidal or insect growth-inhibiting activity into baculoviruses (Wood and Granados, 1991).

Genetic engineering of baculoviruses to insert genes encoding specific toxins, enzymes, or insect hormones that disrupt the development of pest insects when expressed at inappropriate times, may enhance the rate of killing caused by these insecticidal agents. For example, recombinant viruses containing neurotoxin genes exhibit greater insecticidal activity than the wild type viruses (Maeda *et al.*, 1991; Tomalski and Miller, 1991; Stewart *et al.*, 1991; McCutchen *et al.*, 1991).

One protein that has potential insecticidal activity is the enzyme chitinase. Insect chitinase plays a critical role

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in insect growth and development (Kramer and Koga, 1986). The induction of chitinase in response to ecdysteroids facilitates molting. Its substrate, chitin, occurs in the exoskeleton and gut lining of insects. Insects use chitinolytic enzymes to digest chitin during the molting process (Kramer and Koga, 1986). Administration of chitinase to insects at inappropriate times during development could disrupt cuticle and/or gut physiology. Previous evidence indicated that bacterial chitinases increased host mortality caused by an entomopathogenic bacterium and virus (Smirnoff, 1971, 1973; Morris, 1976; Dubois, 1977; Gunner *et al.*, 1985; Shapiro *et al.*, 1987). Recently, we reported the cloning and characterization of a cDNA encoding epidermal and gut chitinases of the tobacco hornworm, *Manduca sexta* (Kramer *et al.*, 1993). That effort was a first step towards introduction of the insect chitinase gene into microbes and plants for testing of the recombinant enzyme as a potential control agent in the management of insect pests and plant pathogens. In this paper, we describe the construction of a recombinant nonoccluded baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), containing the *M. sexta* chitinase cDNA under the control of the polyhedrin promoter. *Spodoptera frugiperda* and *Trichoplusia ni* cells infected with this virus produced and secreted into the culture media an enzymatically active protein with the same apparent molecular mass (85 kDa) as that of chitinase present in *M. sexta* pharate pupal molting fluid. Enzymatically active protein was also present in the hemolymph of *M. sexta* and *S. frugiperda* larvae infected with the recombinant virus. Bioassays demonstrated that the recombinant virus killed *S. frugiperda* larvae more rapidly than a wild type virus, indicating that the recombinant chitinase may be an insecticidal protein that improves the efficacy of the baculovirus.

MATERIALS AND METHODS

Cells and viruses

S. frugiperda cell lines SF9 and SF21 were propagated at 28°C in SF-900 II serum-free insect cell culture medium obtained from GIBCO BRL (Gaithersburg, MD). The *T. ni* Hi-5 cell line (Tn5B1-4) was propagated in EXCELL-400 serum-free medium containing L-glutamine (JRH Biosciences, Lenexa, KS). The transfer vector pVL1393 was from Invitrogen (San Diego, CA). Wild type *A. californica* nuclear polyhedrosis virus (AcMNPV) was obtained from GIBCO BRL and was originally isolated by Dr Edward M. Dougherty, Insect Biocontrol Laboratory, ARS, USDA, Beltsville, MD 20705. Cell culture techniques were as described by Summers and Smith (1987). Viral infection of insect cells was carried out using the BaculoGold Transfection Kit from Pharmingen (San Diego, CA), which employed baculovirus strain C6 that contained a lethal deletion.

Construction of recombinant baculovirus containing the M. sexta chitinase gene

The 9.2 kb baculovirus transfer vector pVL1393 contains the promoter for the polyhedrin gene followed by a polylinker with a unique restriction site for EcoRI. The 1.8 kb EcoRI fragment from *M. sexta* chitinase clone 201 containing the entire coding region (Kramer *et al.*, 1993) was inserted at the EcoRI site of pVL1393 to produce the transfer plasmid pVL1393·chi. All DNA manipulations were done according to standard procedures (Maniatis *et al.*, 1982). Restriction enzyme analysis was performed to identify the construct containing the insert in the correct orientation.

BaculoGold linearized virus DNA (0.5 μg) was mixed with the transfer plasmid pVL1393·chi (2 μg) and transfected into 3×10^6 SF9 cells (Kitts and Possee, 1993). The recombinant baculovirus, vAcMNPV·chi, was amplified, and the medium containing the recombinant virus was used to infect the insect cells. This procedure is expected to yield greater than 99% of the total plaques as recombinants. Because of the presence of a lethal deletion in the viral DNA used for transfection, recombination of the viral DNA with the transfer vector DNA was necessary for the virus to become viable. The plaque phenotype of vAcMNPV·chi was occlusion negative. Budded virus from a wild type baculovirus (vAcMNPV·wt) was used as the control virus.

Western blot analysis

One million cells in 2 ml SF-900 II (SF9 and SF21) or EXCELL-400 (Hi-5) medium were seeded into a 35-mm tissue culture dish for 30 min to 1 h at 28°C. The medium then was removed, and cells were infected at a multiplicity of infection of approximately 20 plaque forming units (PFU) per cell with either vAcMNPV·wt or vAcMNPV·chi for 1 h at room temperature. The viral inoculum was removed, and cells were incubated with 2 ml of cell culture medium at 28°C. Two days after infection, the medium was collected and centrifuged at 12,000 ***g*** for 5 min to remove floating cells; an aliquot of the supernatant was subjected to 3–17% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Jule Biotechnologies Inc., New Haven, CT) (Laemmli, 1970). Cells were suspended in 0.3 ml of 20 mM Tris, 2 mM EDTA and lyzed either by sonication for activity assay or by suspension in 0.1 ml of 2 × SDS-gel loading buffer (4% SDS, 125 mM Tris–HCl pH 6.7, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 2% (v/v) β-mercaptoethanol). Lysates were diluted two-fold prior to electrophoresis. After electrophoresis, proteins from the medium and cell lysate were transferred to a poly(vinylidenedifluoride) (PVDF) membrane with a Semi-Dry Electroblotter (Integrated Separation Systems, Natick, MA) according to the manufacturer's instructions. The membranes were blocked with 2.5% gelatin and then processed as described by Khyse-Anderson (1984). A polyclonal antibody to chitinase from the molting fluid of fifth instar

M. sexta larvae (Koga *et al.*, 1983) was used to detect chitinase-related proteins by immunoblot analysis. Bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG. Western blot analysis also was done with 1–10 μl of hemolymph collected from recombinant, wild type virus-infected, and uninfected larvae 6 days after infection.

Chitinase activity assay

Media and cell lysates obtained two days after infection of insect cells with viruses were subjected to electrophoresis in a native 7.5% polyacrylamide minigel at pH 8.8 (Blackshear, 1984). After electrophoresis, the gel was overlaid with a 7.5% polyacrylamide gel containing 0.1% glycol chitin as substrate and incubated at 37°C for 1.5 h following the procedure of Trudel and Asselin (1989). Chitinase bands were detected as lytic zones by the absence of staining with fluorescent brightener (Sigma Chem. Co., St. Louis, MO) when viewed under ultraviolet light.

Quantitation of chitinase activity was done using a colorimetric assay with carboxymethylchitin Remazol Brilliant Violet (CM–Chitin–RBV) as the substrate (Loewe Biochemica, Nordring, Germany). Samples were diluted with water to 0.4 ml and incubated at 37°C with 0.2 ml of 0.2 M phosphate–citrate buffer pH 7.5 and 0.2 ml of CM–Chitin–RBV for 1 or 2 h. The reaction was stopped by addition of 0.2 ml of 2 N HCl, and samples were cooled on ice for 15 min. After the samples were centrifuged at 12,000 *g* for 5 min, the absorbance at 550 nm of the supernatants was determined.

Glycosylation studies

Tunicamycin (Sigma, St. Louis, MO) was added at a concentration of 5 μg ml^{-1} to the cells 15 h after infection. The cells then were incubated for an additional 33 h, after which the media and cells were separated and analyzed using 3–17% gradient SDS-PAGE, followed by immunoblot analysis using an antiserum to *M. sexta* chitinase.

Carbohydrate analysis of chitinase from molting fluid of fifth instar *M. sexta* larvae was done by subjecting 10 μl of molting fluid to 3–17% gradient SDS-PAGE followed by transfer on to a PVDF membrane. The membrane then was stained with Coomassie brilliant blue R-250 (0.4% in 10% acetic acid) for 5 min, followed by destaining in 80% methanol for 5 min. The protein corresponding to chitinase was identified by treating a duplicate blot with the antibody to chitinase for 3 h. The protein band corresponding to chitinase was cut out from the Coomassie blue-stained membrane, and its carbohydrate composition was determined at the Experimental Station Chemical Laboratories, University of Missouri, Columbia. Samples were hydrolyzed to liberate carbohydrate moieties, and sugars were reduced and derivatized to alditol or hexosaminitol acetates, which were separated by gas–liquid chromatography and quantified by mass spectrometry (Mawhinney *et al.*, 1980; Mawhinney, 1986; Tilley *et al.*, 1993).

Carbohydrate analysis of the chitinase expressed in recombinant baculovirus-infected Hi-5 cells also was done. One milliliter of the culture medium was subjected to 20% trichloroacetic acid precipitation to concentrate the protein, which was suspended in sample buffer and subjected to 3–17% gradient SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (0.2% in 20% methanol, 10% acetic acid). The band corresponding to *M. sexta* chitinase was cut out, and its carbohydrate composition determined as described above.

N-terminal sequencing

Proteins were subjected to SDS-PAGE, electroblotted on to a PVDF membrane, which was then stained with Coomassie brilliant blue. The protein band of interest was cut out and subjected to automated Edman degradation using an Applied Biosystems Sequencer (Matsudaira, 1987; Tempst and Riviere, 1989) at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan.

Insect bioassays

Two different sources of virus were used for the insect bioassays, a wild type virus vAcMNPV·wt from GIBCO BRL and a recombinant virus vAcMNPV·chi constructed from baculovirus strain C6 from Pharmingen. *S. frugiperda* larvae were supplied by Dr Thomas Coudron (Biocontrol Laboratory, ARS-USDA, Columbia, Missouri). Twenty-five to 30 late fourth instar larvae were injected with 2×10^5 PFU of budded virus vAcMNPV·wt or vAcMNPV·chi (Eldridge *et al.*, 1992). Controls were either uninjected larvae or larvae injected with only media. Infected and control insects were reared individually on artificial diet at 28°C on a 14:10 h light:dark cycle. Mortality was recorded every 8 h following infection, and LT_{50} values were determined by probit analysis (Daum, 1970).

RESULTS

Baculovirus expression of M. sexta chitinase in S. frugiperda (SF) cell lines

The recombinant baculovirus vAcMNPV·chi, containing the entire coding region of the *M. sexta* chitinase plus the putative signal peptide (Kramer *et al.*, 1993) under control of the polyhedrin promoter, was constructed as described in Materials and Methods. SF9 cells were infected with either vAcMNPV·chi or the wild type virus, vAcMNPV·wt. Two days after infection, media and lysates from infected and uninfected cells were analyzed by western blotting using an antibody to *M. sexta* chitinase (Koga *et al.*, 1983) (Fig. 1A). An immunoreactive protein of approximately 85 kDa occurred in both the lysate (lane 4) and medium (lane 7) from cells infected with the recombinant virus. This band was absent in medium and lysate from wild type virus-infected and uninfected cells (lanes 2, 3, 5 and 6).

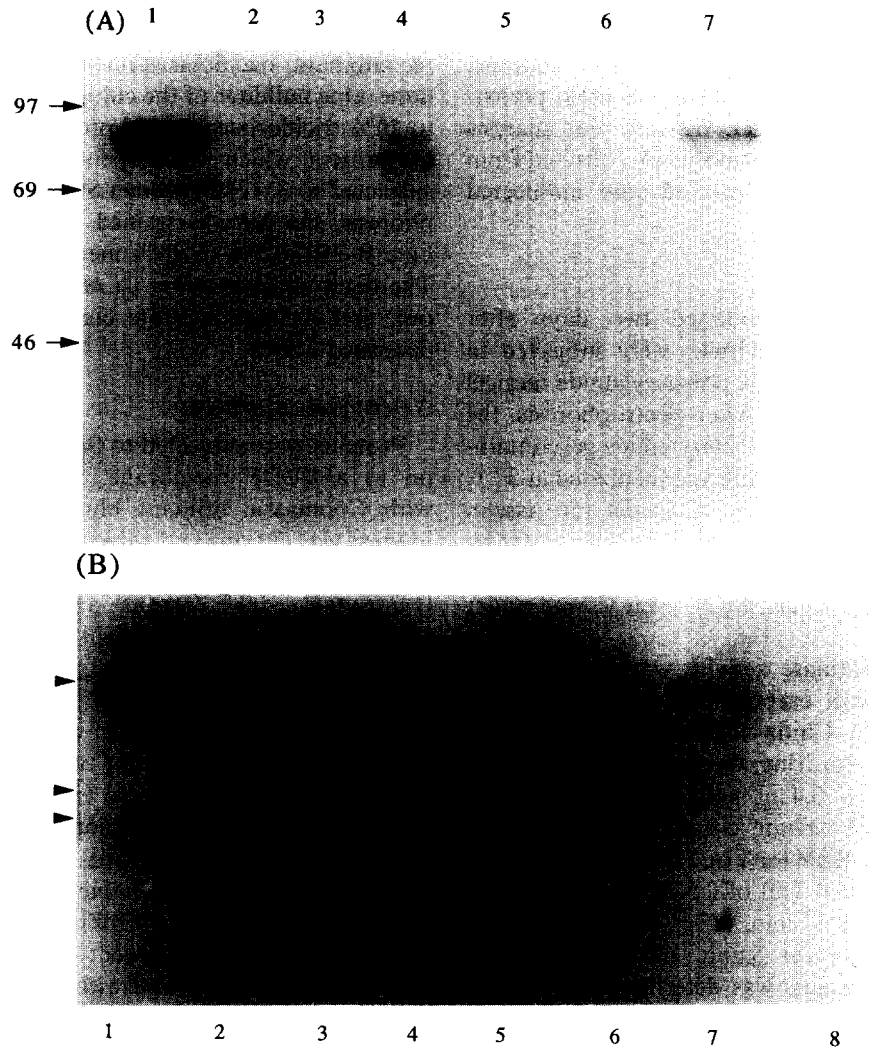

FIGURE 1. Expression of *Manduca sexta* chitinase in baculovirus-infected SF9 cells. 10^6 cells were infected with vAcMNPV·wt or vAcMNPV·chi virus at a multiplicity of infection of approximately 20. Uninfected cells were used as control. Two days after infection, media were harvested, and cells were lyzed. (A) Aliquots containing 60 and 25 μg of protein from the medium and cell lysate, respectively, were analyzed by SDS-PAGE followed by immunoblotting with antibody to *Manduca sexta* chitinase. The molecular weights of size marker proteins are given in kDa. Lane 1, molting fluid (1 μl); lane 2, lysate from uninfected cells; lane 3, lysate from wild type virus-infected cells; lane 4; lysate from recombinant virus-infected cells; lane 5, medium from uninfected cells; lane 6, medium from wild type virus-infected cell; and lane 7, medium from recombinant virus-infected cells. (B) 25 μg of protein was used for chitinase activity assay. The positions of chitinases are indicated by arrow heads. Lanes 1 and 5, molting fluid (1 μl); lane 2, lysate from wild type virus-infected cells; lane 3, lysate from recombinant virus-infected cells; lane 4, lysate from uninfected cells; lane 6, medium from wild type virus-infected cells; lane 7, medium from recombinant virus-infected cells; and lane 8, medium from uninfected cells.

The mobility of the immunoreactive protein after SDS-PAGE was the same as that of chitinase present in molting fluid of fifth instar *M. sexta* larvae (lane 1), indicating that the molecular weights of the two proteins were identical. The cell lysates also contained a smaller immunoreactive protein (approximately 80 kDa), which was more abundant than the 85 kDa protein. However, only the slower migrating 85 kDa protein was detected in the medium. Thus, *M. sexta* chitinase-like proteins were present not only in recombinant virus-infected cells, but also in the medium, where the largest of the immunoreactive proteins was found.

Following native gel electrophoresis, aliquots of the cell extracts and media also were used for chitinase assays with glycol chitin as the substrate (Trudel and Asselin, 1989) (Fig. 1B). The lysate (lane 3) and medium (lane 7) from recombinant virus-infected SF9 cells contained a protein with chitinase activity that had the same mobility ($R_m = 0.52$) as the chitinase present in molting fluid (lanes 1 and 5). This protein was absent from lysates and media from control and wild type virus-infected cells (lanes 2, 4, 6 and 8). Two other chitinases with higher mobility ($R_m = 0.67$ and 0.71) also were observed in both the wild type and recombinant virus-infected cells. Presumably, these proteins were chitinolytic enzymes endogenous to the virus (Maeda, S., personal communication). The viral chitinases were detected at lower levels than the recombinant chitinase, did not cross-react with the *M. sexta* chitinase antibody, and were not present in molting fluid (lanes 1 and 5)

or in uninfected cell extracts and media (lanes 4 and 8). Because no immunoreactive protein was detected by western blot analysis in wild type virus-infected cells (lane 3) and media (lane 6) by the *M. sexta* chitinase antibody, the viral chitinolytic enzymes are immunologically unrelated to *M. sexta* chitinase.

The time course of expression for *M. sexta* chitinase in SF9 cells infected with the vAcMNPV·chi virus was determined by immunoblotting. Expression of immunoreactive chitinase was observed in the cell lysate after 8 h p.i. (Fig. 2, lane 11). The major protein in the cell lysate was approximately 80 kDa in size. It appeared after about 32 h and increased with time thereafter. The most abundant chitinase that was found in the medium, which was about 85 kDa in size, exhibited a dramatic increase in expression level from 32 to 40 h, and continued to increase at later time points. An immunoreactive protein of apparent molecular mass of about 52 kDa also was detected at about 40 h and increased in amount with time thereafter (Fig. 2, lanes 5–9, 15–19). An immunoreactive 52 kDa protein was sometimes detected in molting fluid, which may be a proteolytic breakdown product of the 85 kDa chitinase.

The recombinant M. sexta chitinase secreted into the medium is glycosylated

The multiple immunoreactive proteins in cell lysates and media (Fig. 2) and the difference between the apparent molecular weights of the recombinant enzyme (85 kDa) and that of the primary translation product of *M. sexta* chitinase mRNA in a reticulocyte lysate (75 kDa, Kramer *et al.*, 1993) led us to investigate whether these observations were due to glycosylation. *M. sexta* chitinase has 4 potential *N*-linked glycosylation sites in its amino acid sequence (Kramer *et al.*, 1993). Expression of chitinase in recombinant virus-infected SF9 cells from 16 to 48 h p.i. in the presence or absence of tunicamycin, an inhibitor of *N*-linked glycosylation of protein, is shown in Fig. 3. In the absence of tunicamycin, immunoreactive chitinase was released into the medium as an 85 kDa protein (Fig. 3, lane 3). However, tunicamycin inhibited the secretion of this protein into the medium (lane 4). In extracts of cells not treated with tunicamycin, the antibody to *M. sexta* chitinase cross-reacted with a major protein of approximately 80 kDa. A small amount of the 85 kDa protein also was detected (lane 1). Cells treated with tunicamycin exhibited an immunoreactive protein with an apparent molecular weight of only about 75 kDa (lane 2). The slower migrating 80 kDa protein from the cells not treated with tunicamycin was apparently a partially glycosylated form of chitinase. *N*-linked glycosylation appears to be required for secretion of the protein into the medium, because in the presence of tunicamycin, no immunoreactive protein was found in the medium and the smallest immunoreactive protein (75 kDa) was found in cell extracts.

The carbohydrate compositions and N-terminal amino acid sequences of the 85 kDa chitinases isolated from molting fluid and the culture medium from recombinant virus-infected cells were determined. The carbohydrate contents were similar, consisting of glucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, mannose and galactose, which together accounted for approximately 25% of the mass of enzymes (Table 1). The first six N-terminal amino acid residues of both proteins were found to be identical, DSRARI, indicating that the molting fluid and recombinant chitinases have undergone identical N-terminal processing.

Chitinase expression in SF9, SF21 and Hi-5 cell lines

A comparative immunoblot analysis of the expression of recombinant chitinase in SF9, SF21 and Hi-5 cell

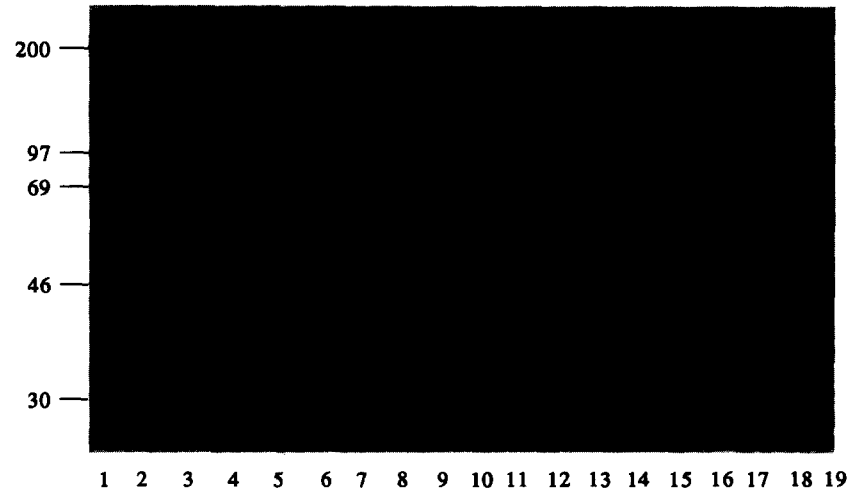

FIGURE 2. Time course of expression of *Manduca sexta* chitinase in SF9 cells. 10^6 cells were infected with vAcMNPV·chi virus. Medium and cell lysate were collected at 8-h intervals up to 72 h p.i. 100 μl of medium and 2 μl of cell lysate were analyzed by SDS-PAGE followed by western blot analysis with antibody to *M. sexta* chitinase. Lanes 1–9, media from infected cells harvested at 8, 16, 24, 32, 40, 48, 56, 64 and 72 h, respectively. Lanes 11–19, cell lysates from infected cells collected at 8, 16, 24, 32, 40, 48, 56, 64 and 72 h, respectively. Lane 10, molting fluid.

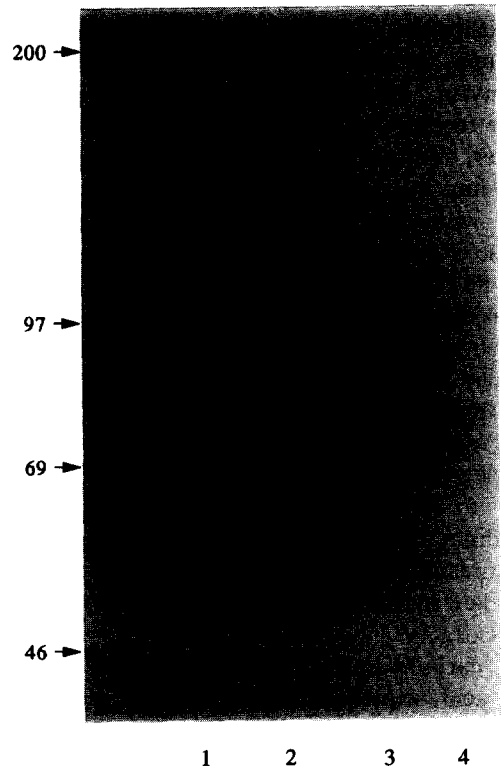

FIGURE 3. Effects of tunicamycin on expression of *Manduca sexta* chitinase. SF9 cells were infected with vAcMNPV·chi and incubated at 28°C. Tunicamycin (5 μg/μl) was added 15 h after infection, and incubation at 28°C was continued for an additional 33 h. Infected cells not containing tunicamycin served as controls. Media and cell lysates were harvested, and 100 μl and 5 μl, respectively, of each were analyzed by SDS-PAGE followed by immunoblot analysis using *M. sexta* chitinase-specific antisera. Lane 1, lysate from cells without tunicamycin treatment; lane 2, lysate from cells treated with tunicamycin; lane 3, medium from cells treated without tunicamycin; and lane 4, medium from cells treated with tunicamycin.

lines infected with the vAcMNPV·chi virus revealed that the relative yields of immunoreactive protein were in the order Hi-5 (Fig. 4A, lanes 2 and 5) > SF21 (lanes 3 and 6) > SF9 (lanes 4 and 7), both in the cell lysates and media. Coomassie brilliant blue staining of the gel of the medium from the Hi-5 cells revealed that the major protein had the same mobility as the molting fluid chitinase (Fig. 4B). This protein also cross-reacted with the chitinase antibody (data not shown). The levels of chitinase activity in media from vAcMNPV·wt- and vAcMNPV·chi-infected or mock-infected SF9, SF21 and Hi-5 cell lines also were determined (Table 2). Medium from Hi-5 cells contained nearly seven-fold and three-fold more chitinase activity than media from SF9 and SF21 cells, respectively. Media from vAcMNPV·wt-infected and mock-infected cells contained little or no enzyme activity.

TABLE 1. Carbohydrate composition of *M. sexta* wild-type and recombinant chitinases*

	Chitinase†	
Carbohydrate	Native	Recombinant
Glucose	9.0	14.6
N-Acetylgalactosamine	8.2	3.7
N-Acetylglucosamine	3.1	2.1
Mannose	2.7	2.9
Galactose	0.9	4.0
Total	23.9	27.3

*Values were determined by GC-mass spectrometry and are expressed as percentages of sample weight.

†The native enzyme was isolated from pharate pupal molting fluid and the recombinant protein from a baculovirus-insect cell line (Hi-5) gene expression system.

Baculovirus expression of chitinase in vivo

To determine whether the vAcMNPV·chi virus expresses chitinase in larvae, fourth instar *M. sexta* larvae were infected with 0.6×10^6 PFU of either the vAcMNPV·wt or vAcMNPV·chi virus. Hemolymph was collected from larvae 6 days after infection and monitored for the presence of recombinant protein by immunoblotting (Fig. 5A). An immunoreactive protein of about 85 kDa in size was detected in hemolymph from vAcMNPV·chi virus-infected larvae (lane 1). Neither control hemolymph (lane 3) nor hemolymph from larvae infected with the vAcMNPV·wt virus (lane 2) contained any immunoreactive protein. The size of the recombinant protein (lane 1) was the same as the chitinase present in molting fluid of fifth instar *M. sexta* larvae (lane 4).

Chitinase activity was measured following native gel electrophoresis of hemolymph from *M. sexta* larvae infected with the vAcMNPV·chi virus (Fig. 5B). Hemolymph contained a chitinase (lane 2) that had the same mobility as *M. sexta* molting fluid chitinase (lane 1).

To determine whether recombinant chitinase was expressed *in vivo* in *S. frugiperda* larvae, fourth instar larvae were injected with 2×10^5 PFU of either vAcMNPV·chi or vAcMNPV·wt virus. Hemolymph was collected from the infected and control larvae at 24 h intervals p.i. and subjected to western blot analysis (Fig. 6A). Recombinant protein was detected in the hemolymph of *S. frugiperda* larvae infected with the recombinant virus, as early as 24 h after infection (lane 4) and continued to increase at 48 and 72 h (lanes 6 and 8). The mobility of the recombinant protein was identical to that of chitinase present in molting fluid of *M. sexta* larvae (lane 11). Hemolymph from control larvae (lanes 3 and 10) and wild type virus-infected larvae (lanes 2, 5, 7 and 9) did not contain immunoreactive protein. The 52 kDa immunoreactive protein also observed apparently is a proteolytic product derived from the larger immunoreactive protein. For comparison, molting fluid exhibited similar immunoreactive protein patterns, if precautions were not taken to prevent proteolysis (Fig. 2).

Hemolymph from larvae bled 3 days p.i. was used for chitinase assay using glycol chitin as the substrate (Fig. 6B). The mobilities of the proteins with chitinase

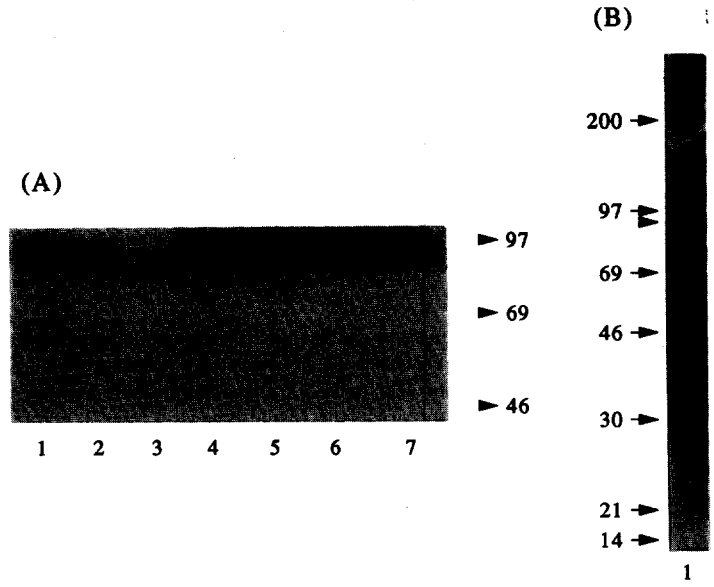

FIGURE 4. (A) Expression of *Manduca sexta* chitinase in infected cell lines. 10^6 Hi-5, SF21, or SF9 cells were infected with vAcMNPV·chi virus at a multiplicity of infection ~20 pfu/cell in 2 ml of medium and incubated at 28°C for 2 days. Media and cell lysates were harvested thereafter. 75 μl of medium (3.75% of total) and 3 μl of lysate (1% of total), respectively, were analyzed by SDS-PAGE, subjected to electroblotting and probed with antibody to *M. sexta* chitinase. Lane 1, molting fluid; lane 2, cell lysate from Hi-5; lane 3, cell lysate from SF21; lane 4, cell lysate from SF9; lane 5, medium from Hi-5; lane 6, medium from SF21; and lane 7, medium from SF9. (B) Coomassie brilliant blue R-250 staining of SDS-PAGE of 200 μl medium from Hi-5 cells two days after infection. The arrow head indicates the position of the major protein with a mass of 85 kDa.

activity in molting fluid and recombinant virus-infected larvae were identical ($R_m = 0.38$). As was observed in cell culture extracts (Fig. 1B), high mobility proteins with chitinase activity were detected in hemolymph from both wild type and recombinant virus-infected larvae (Fig. 6B, lanes 2 and 3, respectively). These proteins were probably viral-derived chitinolytic enzymes.

Finally, we compared the levels of chitinase expressed in three baculovirus-infected cell lines and two larvae with the level measured in *M. sexta* pharate pupal molting fluid (Table 3). When compared on a per mg basis, virus-infected armyworm hemolymph had the highest activity followed by hornworm molting fluid, Hi-5 cell culture medium, and hornworm hemolymph.

TABLE 2. Chitinase expression in media from baculovirus infected cell lines

Cell line	Sample	Activity* (ΔA_{550}/h/ml medium)
SF9	Uninfected	0.19
	vAcMNPV·wt	0.16
	vAcMNPV·chi	0.67
SF21	Uninfected	0.28
	vAcMNPV·wt	0.25
	vAcMNPV·chi	1.95
Hi-5	Uninfected	0.29
	vAcMNPV·wt	0.45
	vAcMNPV·chi	4.80

*Activity was determined colorimetrically using CM–chitin–RBV as substrate.

The latter two fluids had only about 15% and 30%, respectively, of the activity of armyworm hemolymph, whereas the activity of molting fluid was about 60%. A six-fold higher level of chitinase activity occurred in *S. frugiperda* hemolymph than in *M. sexta* hemolymph, a result consistent with the observation that the former species is more susceptible to the virus than the latter. On an equal volume basis, *S. frugiperda* hemolymph was approximately 60% as active as *M. sexta* molting fluid.

Effect of viral infection on mortality of S. frugiperda larvae

To determine whether insertion of the *M. sexta* chitinase gene into the AcMNPV genome would enhance the insecticidal activity of the baculovirus, mortality of fourth instar *S. frugiperda* larvae infected with the recombinant or wild type virus was monitored as a function of time (Fig. 7). Larvae infected with the vAcMNPV·chi virus died about 1 day earlier than those infected with the wild type virus. The LT_{50} values, which are the times at which 50% of the larvae had died, were compared between recombinant and wild type virus-infected larvae. The LT_{50} for insects infected with the recombinant virus was approximately 18–20 h shorter than that observed for wild type virus-infected insects. The LRT_{50} value, which is the ratio of times when 50% of the larvae had died after infection with either the recombinant or wild type virus (Bonning and Hammock, 1993), was 0.77–0.78, demonstrating that the recombinant virus killed larvae in approximately 75% of the

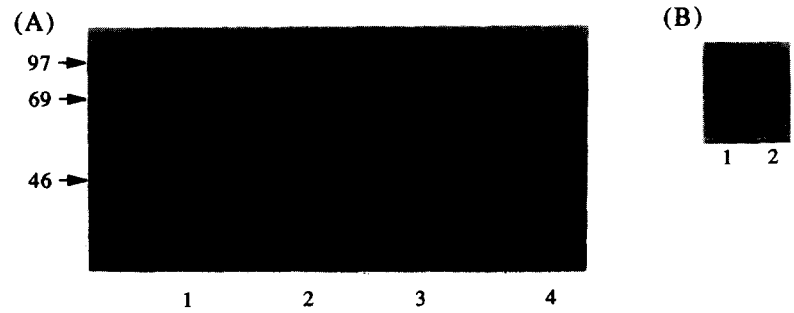

FIGURE 5. Western blot analysis (A) and chitinase activity gel analysis (B) of hemolymph of fourth instar *Manduca sexta* larvae infected with vAcMNPV·wt or vAcMNPV·chi virus. Hemolymph was collected from recombinant or wild type virus-infected or uninfected larvae 6 days after infection. Ten and 5 μl of hemolymph were used for western blot analysis and activity gel assay, respectively. (A) Lane 1, recombinant virus; lane 2, wild type virus; lane 3, uninfected, and lane 4, molting fluid. (B) Lane 1, molting fluid; and lane 2, recombinant virus.

time period required for the wild type virus to cause the same degree of mortality (Table 4).

DISCUSSION

A recombinant baculovirus with a 1.8 kb EcoRI fragment containing the entire coding region and a 19-amino acid-long leader peptide of *M. sexta* molting fluid chitinase fused to the polyhedrin promoter of *A. californica* nuclear polyhedrosis virus was constructed. Even though this fragment is missing most of the 3′-untranslated region of the chitinase gene, insect larvae and insect cells infected with this recombinant virus expressed and secreted the active enzyme into the

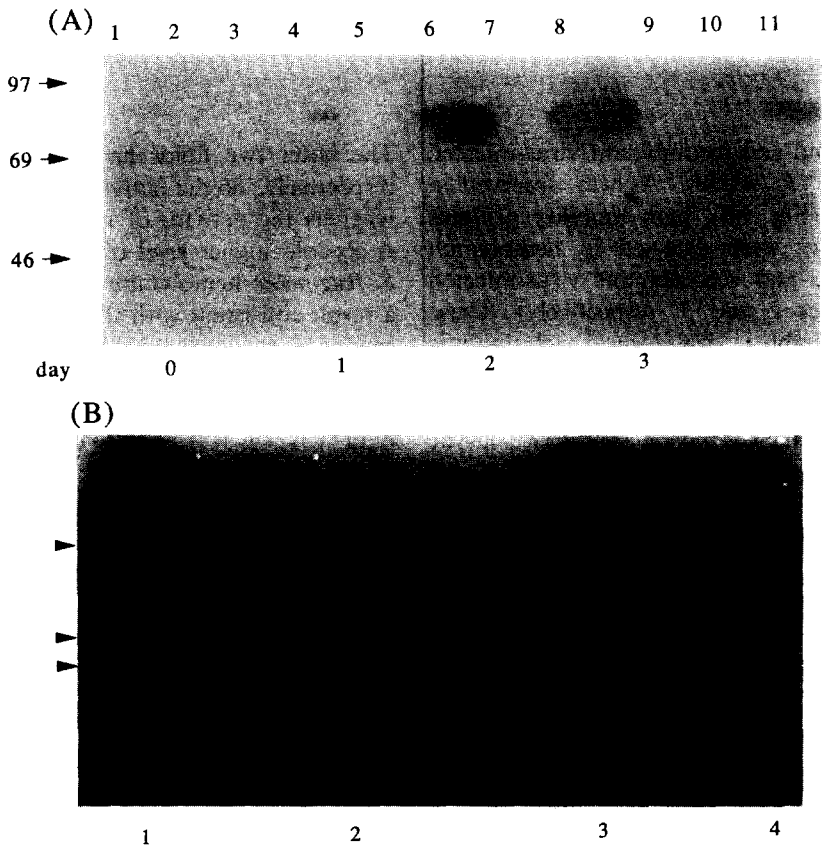

FIGURE 6. Time course of expression of chitinase (A) and chitinase activity (B) in hemolymph of fourth instar *Spodoptera frugiperda* larvae infected with vAcMNPV·wt or vAcMNPV·chi virus. (A) Hemolymph was collected at 24-h intervals, and 10 μl aliquots were analyzed by SDS-PAGE followed by immunoblot analysis with an antiserum to *M. sexta* chitinase. Lanes 1–3, day 0 after infection with vAcMNPV·chi (lane 1); vAcMNPV·wt (lane 2); uninfected (lane 3); lanes 4–5, 1 day after infection with vAcMNPV·chi (lane 4); vAcMNPV·wt (lane 5); lanes 6–7, 2 days after infection with vAcMNPV·chi (lane 6); vAcMNPV·wt (lane 7); lanes 8–10, 3 days after infection with vAcMNPV·chi (lane 8); vAcMNPV·wt (lane 9); uninfected (lane 10); and lane 11, molting fluid (2 μl). (B) Two μl of hemolymph collected from fifth instar *S. frugiperda* larvae 3 days after infection were analyzed for chitinase activity using glycol chitin as a substrate. Arrows indicate the chitinases. Lane 1, molting fluid (2 μl); lanes 2 and 3, hemolymph 3 days after infection with vAcMNPV·wt and vAcMNPV·chi, respectively; and lane 4, uninfected.

TABLE 3. Chitinase activity in hemolymph and medium from baculovirus infected larvae and Hi-5 cell line

Sample	Activity* (ΔA_{550}/h/mg protein)
Spodoptera frugiperda hemolymph	4.21 ± 0.16
Manduca sexta hemolymph	0.67 ± 0.08
Hi-5 cell line medium	1.26 ± 0.01
Molting fluid	2.56 ± 0.32

*Mean value ± 0.5 range ($n = 2$). Substrate = CM–chitin–RBV.

hemolymph and culture medium, respectively. Armyworm hemolymph exhibited six-fold higher levels of chitinase activity than hornworm hemolymph. The levels of chitinase expression also varied among the three insect cell lines tested, with the highest levels being attained in *T. ni* Hi-5 cells. The period of maximum chitinase expression coincided with that of late viral protein synthesis. This finding is consistent with the expected pattern of expression of genes under control of the polyhedrin promoter, which is active late in viral replication.

The molecular mass of the recombinant chitinase (85 kDa) secreted by insect cells and tissues was the same as that of the major chitinase present in molting fluid. This size was larger than either the calculated molecular weight of the protein encoded by the open reading frame of the chitinase clone (62 kDa) or the apparent molecular weight of the major protein (75 kDa) found in translation products of *M. sexta* larval mRNA hybrid-selected by the chitinase cDNA clone 10 with the 1.8 kb EcoRI fragment (Kramer *et al.*, 1993). Previously, we concluded that the *M. sexta* chitinase migrated anomalously in an SDS-PAGE system, but we did not know the cause (Kramer *et al.*, 1993). The finding that the 1.8 kb EcoRI fragment in the recombinant virus directed the synthesis of an 85 kDa protein *in vitro* by insect cells

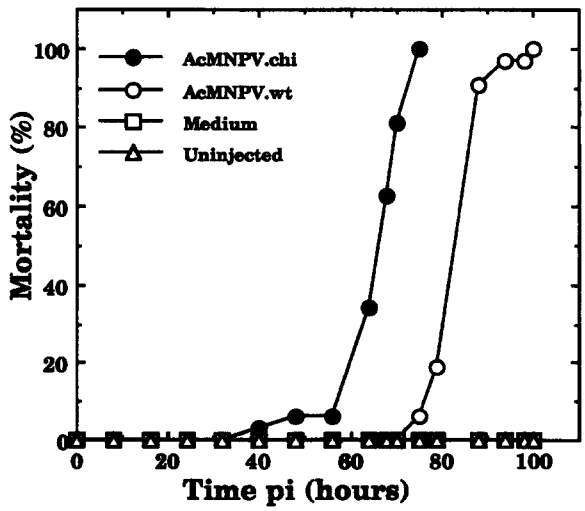

FIGURE 7. Effect of infection with vAcMNPV·wt and vAcMNPV·chi virus on mortality of fourth instar *Spodoptera frugiperda* larvae. 2×10^5 PFU of virus were injected per larva. Thirty-two larvae in each group were monitored at 8-h intervals.

TABLE 4. Effect of wild-type and recombinant *M. sexta* chitinase gene-containing baculovirus on mortality of *Spodoptera frugiperda**

Experiment	Virus type	LT_{50}† (h)	LRT_{50}‡
1	Recombinant	68 (65–71)	0.77
	Wild-type	88 (85–94)	
2	Recombinant	65 (64–67)	0.78
	Wild-type	83 (81–85)	

*Infection of fourth instar larvae with 2×10^5 PFU of vAcMNPV chi or vAcMNPV·wt. Treatments were significantly different at the $\alpha = 0.05$ level ($n = 13$ and 14 for recombinant and wild-type virus, respectively, in experiment 1 and $n = 32$ in experiment 2; Fisher exact test).

†Time after infection when 50% mortality occurred as determined by probit analysis. 95% confidence limits given in parentheses.

‡Ratio for lethal response times when 50% mortality occurred in insects infected with recombinant and wild-type viruses.

indicates that the *M. sexta* chitinase undergoes post-translational modifications. Consistent with this notion are the observations that (1) the molting fluid chitinase is glycosylated (Koga and Kramer, 1983) and (2) the 85 kDa chitinase was not detected *in vitro* in the presence of tunicamycin in either the media or the cells. Instead, a 75 kDa protein was detected by the chitinase antibody, which accumulated in the cells, indicating that secreted enzyme was glycosylated. Indeed, carbohydrate analysis of the recombinant enzyme showed that its carbohydrate content was similar to the molting fluid enzyme. Comparison of N-terminal sequences also demonstrated that the recombinant enzyme had the same N-terminal amino acid sequence as the molting fluid enzyme.

Relatively high-level expression of active chitinase in insect cell lines prompted us to test for effects of baculovirus-mediated expression in *M. sexta* larvae *in vivo*. Preliminary experiments with late fourth instar larvae failed to show enhanced mortality (data not shown), presumably due to the low efficiency of multiplication of AcMNPV in this host. On the other hand, AcMNPV multiplied well in *S. frugiperda* larvae, as shown by the accumulation of high levels of chitinase in its hemolymph. When injected into *S. frugiperda* fourth instar larvae, the recombinant virus killed all of the larvae in approximately 75 h, whereas the wild type virus required about 100 h to cause 100% mortality. These results indicate that chitinase, by increasing the killing rate of insect pathogens, might be useful as a biological control-enhancing agent.

The mechanism of the apparent chitinase toxicity was not determined. Normally, chitinase is produced in molting fluid and gut tissue subsequent to feeding behavior at the end of a larval instar in preparation for a molt (Fukamizo and Kramer, 1987; Kramer *et al.*, 1993). Infection with the recombinant chitinase baculovirus caused expression of the enzyme prematurely and inappropriately in the hemolymph and probably other tissues when the larvae were feeding. Whether this

premature expression leads to a damaged cuticle or peritrophic membrane remains to be determined. Besides cuticle- and peritrophic membrane-associated chitins, which are attacked by chitinase, the presence of additional target compounds in other larval tissues and/or body fluids, remains to be proven. It is possible that oligomeric/polymeric saccharides, which contain multiple *N*-acetylglucosamine residues bonded by $\beta(1\rightarrow4)$ linkages, such as those found in some glycoproteins, are potential substrates.

Because chitinase is readily degradable in the environment and its primary substrate, chitin, does not occur in most microorganisms, plants, or higher animals, the enzyme should be a selective insect-control protein and quite safe for the environment. Furthermore, the possibility of insects developing resistance to chitinase may be relatively low, because molting-associated chitinolysis is essential for larval development and two of the target sites for the enzyme, cuticle and peritrophic membrane, are support structures that are acellular and directly accessible to the enzyme when delivered topically or orally. These structures are relatively unprotected by tissues capable of generating detoxifying mechanisms.

Previously, it was demonstrated that chitinase enhanced the activity of other microbial insecticides. Spruce budworm, *Choristoneura fumiferana*, and gypsy moth, *Lymantria dispar*, larvae died more rapidly when exposed to a bacterial chitinase–*Bacillus thuringiensis* (Bt) mixture than did larvae exposed to the enzyme or bacterium alone (Smirnoff, 1973, 1974; Morris, 1976; Dubois, 1977; Gunner *et al.*, 1985). The insecticidal activity of a nuclear polyhedrosis virus for the gypsy moth was increased five-fold when bacterial chitinase was co-administered (Shapiro *et al.*, 1987). Our results are consistent with those studies and indicate that recombinant insect chitinase also can enhance the activity of microbial insecticides.

Recombinant viral insecticides have been developed that express insect-specific toxins, insect hormones, and an insect-specific enzyme (Hammock *et al.*, 1993). Baculovirus-mediated expression of mite or scorpion toxins in lepidopteran insects generally resulted in paralysis and more rapid death of larvae relative to larvae infected with wild type baculoviruses. When larvae were fed extracts from insect cells infected with a recombinant baculovirus expressing *Bacillus thuringiensis* δ-endotoxin, a reduction in feeding activity and an accelerated mortality occurred (Martens *et al.*, 1990). The genes for two insect hormones, diuretic hormone (Maeda, 1989) and eclosion hormone (Eldridge *et al.*, 1992), were engineered into baculoviruses, but only expression of the former hormone increased the insecticidal activity of the recombinant virus. The first insect-specific enzyme expressed via a recombinant virus was juvenile hormone esterase (Eldridge *et al.*, 1992). However, no difference in developmental characteristics or time of mortality were observed between insects infected with the virus containing the enzyme gene or the control virus, except when neonate larvae were fed a budded form of the virus (Hammock *et al.*, 1990). On the other hand, infection of larvae with a baculovirus in which the virus-encoded ecdysteroid glucosyl transferase (*egt*) gene had been deleted resulted in a slower weight gain and accelerated mortality (O'Reilly and Miller, 1992). Thus, introduction of a gene encoding an enzyme that alters hormonal levels can enhance the insecticidal activity of biocontrol agents. Likewise, our results demonstrate that a gene encoding an enzyme that digests an insect structural component, chitin, can manifest the same effect.

In some of the studies mentioned above, the viruses were packaged as polyhedra and administered orally (Stewart *et al.*, 1991; Tomalski and Miller, 1991). In others, recombinant baculoviruses were tested by injecting larvae with viral suspensions (Maeda *et al.*, 1991; Korth and Levings, 1993). We administered a nonoccluded form of the recombinant virus by injection and showed that chitinase was expressed *in vivo*. Apparently, the recombinant virus had an enhanced insecticidal effect on *S. frugiperda* larvae, killing them approximately 1 day earlier than a wild type virus. However, since the wild type virus and the parent of the recombinant virus were obtained from different sources, our data do not rigorously demonstrate that expression of the chitinase gene would improve the properties of the virus as a pesticide. To do so would require feeding studies using the occluded recombinant virus and the corresponding parent virus. We did not test our recombinant virus orally, because it lacks the polyhedrin gene and only the occluded form of the virus packed in polyhedra can infect via the gut.

Although baculoviruses have been proposed as alternatives to synthetic chemical pesticides for over 20 years, only limited usage has occurred (Wood and Granados, 1991). Recent efforts to improve viral pesticides via genetic enhancement have been successful enough to warrant additional studies aimed at overcoming their limitations. We are now constructing an occluded form of the recombinant chitinase baculovirus that will be tested for insecticidal activity when orally administered.

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